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14. ABSTRACT: The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens and to study the molecular pathogenesis of rickettsioses. The following specific aims were proposed: 1) To establish heterogeneous cell populations, with each cell expressing a unique member of a complex combinatorial peptide-based (e.g., adaptein) library and challenge with <i>R. prowazekii</i> , <i>R. rickettsii</i> , and <i>O. tsutsugamushi</i> ; 2) To determine the role of NF-κB, cytokines, ROS and NO in intracellular killing of rickettsia-infected monolayers containing adapteins and 3) To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability. During the fourth year of the project, rickettsial challenges performed with SV-HCEC cells were continued and expansion of the "resistant colonies" was not possible. We are currently conducting experiments with a murine and a human monocytic cell line that grow in suspension. Both cell lines have been infected with pantropic retroviruses and challenging experiments are underway. Experiments with SVHCEC cells have elucidated the role of rickettsiae and cytokines in modulating permeability across infected monolayers. Confocal microscopy studies also suggest that these changes might in part be due to changes in p120 distribution in adherens junctions. The role of nitric oxide and its derivative peroxynitrite in increased permeability across infected monolayers has also been elucidated.					
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Table of Contents

COVER.....

SF 298.....2

Table of contents.....4

Introduction.....5

Key Research Accomplishments.....17

Reportable Outcomes.....17

Conclusions.....18

References.....19

Appendices.....21

I. INTRODUCTION

Rickettsiae are obligately intracellular organisms that have evolved in close association with an arthropod host. Diseases caused by these organisms are still prevalent in many parts of the world and include Rocky Mountain spotted fever (the most common rickettsiosis in the US), epidemic and endemic typhus (1-4). The latter two are still responsible for thousands of deaths around the world every year.

Rickettsia prowazekii and *R. rickettsii* are listed in the Select Agents Act and are part of the Centers for Disease Control and Prevention (CDC) and NIH category B agents and the North Atlantic Treaty Organization (NATO) select agent list for their potential use as bioterrorist/biowarfare agents (5-8). The most feared complications of rickettsial infections are the development of severe cerebral and pulmonary edema leading to permanent neurologic sequelae or death owing to respiratory failure (3,4).

The target cell of rickettsial pathogens is the endothelium lining the vessels of the microvasculature, as demonstrated by studies performed on autopsy cases dying of rickettsioses and animal models (9,10). The purpose of this study is to utilize adaptein libraries coded within pantropic retroviral vectors that confer protection against rickettsial pathogens. In addition, molecular pathogenesis of rickettsioses is being studied by developing *in vitro* models to study endothelial permeability and intracellular rickettsial killing in both wild type and adaptein protected cells. The long term objective of this proposal is to develop new treatments for rickettsioses and to identify novel molecular targets of rickettsial pathogenesis that would provide sites for new therapeutic interventions, and to eventually use these targets to develop effective and rapid BWT countermeasures. The new therapeutic interventions are

justified due to the narrow range of antimicrobial agents available for rickettsiae and *Orientia*, emergence of chloramphenicol- and tetracycline-resistant strains of *Orientia*, and the possibility of genetically engineered resistance.

II. RESEARCH PROGRESS

During the third year of funding for this project, we have been able to continue experiments related to all specific aims.

Specific aim # 1: To utilize retrovirally encoded adapteins to generate cell monolayers resistant to rickettsial challenge.

As mentioned in our previous progress report (February, 2005), attempts at expanding EGFP-resistant colonies were unsuccessful. During the fourth year of the project, we continued with those attempts and they were unsuccessful. During this past year we have successfully induced maturation of a murine monocytic cell line (M1) to macrophage phenotype which still grows in suspension, therefore facilitating cell sorting based on viability and EGFP expression. Cell suspensions of M1 cells at concentrations of 1×10^7 cells/ml were grown in T25 flasks and exposed to dexamethasone (10^{-5} M). Expression of macrophage surface markers was evaluated using FITC-labeled antibodies against CD11b. Cell suspensions were then analyzed by flow cytometry. Expression of CD11b was detected in > 95% of cells (Figure 1). The differentiated M1 cells were then infected with pantropic retroviruses containing the EGFP scaffold. The percentage of successfully infected cells was >95% in experiments performed in triplicate (Figure 2). Challenging experiments followed by sorting based on EGFP expression and propidium iodide exclusion (as evidence of viability) with *R. rickettsii* are currently underway. Another cell line being currently

optimized for these experiments is a human monocytic leukemia cell line (THP-1) that also grows in suspension. We are evaluating its potential to support rickettsial growth and maturation induction by using vitamin D3, retinoic acid or phorbol esters.

Specific aims #2 and #3: To determine the roles of NF- κ B, cytokines, ROS and NO in intracellular killing of rickettsia-resistant monolayers. To characterize signal transduction pathways modulating the cytoskeletal events responsible for the increased vascular permeability seen in rickettsial infections

Experiments related to specific aims #2 and #3 during the fourth year of the project have continued with the use of sarcoma virus-transformed human brain microvascular endothelial cells (SV-HCEC) and murine primary microvascular endothelial cells obtained by a protocol described in detail in the previous progress report.

Based on the results obtained during the third year regarding increased permeability in endothelial cell monolayers due to rickettsial organisms and the modulating effect of three cytokines evaluated (TNF- α , IL-1 β , and IFN- γ), we expanded the experiments using different MOI of *R. rickettsii* and a wider range of cytokine concentrations (from 0.1 to 1,000 ng/ml).

1. Effects of rickettsiae on SV-HCEC monolayers

SV-HCEC monolayers were infected with 1, 10, 20, and 50 MOI of *R. rickettsii* and the electrode arrays were then connected to the ECIS detection system. Control cells were treated with SPG buffer which was used to resuspend the renografin purified rickettsiae.

Confluent SV-HCEC cells infected with *R. rickettsii* exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in resistance (Figure 3). Resistance recorded using SV-HCECs declined steadily over time after rickettsiae were internalized. At 24 hours, increases in permeability ranged from 12% at 1 MOI to 25% at 50 MOI. At 48 hours post-infection, monolayers infected with 1 MOI showed a 25% increase in permeability. During the first 24 to 48 hours increases in permeability were more pronounced in monolayers infected with 20 and 50 MOI as opposed to 1, 5 and 10 MOI. Differences between these two groups were less evident at 72, 96 and 120 hours of infection, most likely reflecting similar cell death rates in all monolayers at late time points. By the end of the experiment at 120 hours, all monolayers showed a 50-55% increase in permeability when compared to the controls.

2. Cell death rates in rickettsiae-infected SV-HCEC monolayers

In order to ascertain the role of cell death in increased permeability across the endothelial monolayers death cell curves were performed. SV-HCEC monolayers were grown to confluency in 35mm plastic Petri dishes and infected with 15 MOI of *R. rickettsii*. At 24, 48 and 72 hours, the supernatants were aspirated and the monolayers were stained using the Live-Dead Viability Stain (Molecular Probes, Eugene, OR). Images from fields were obtained using an FV-1000 Confocal Microscope and a 10x objective. Propidium iodide (PI) uptake by cells was used to calculate the percent of cells that were undergoing necrotic cell death. Quantification of cell death was performed by determining the percent of total cells staining positive for PI. Cell death rates did not reveal any differences between infected and non-

infected monolayers up to 48 hours post-infection (Figure 4), suggesting that increases in permeability seen during the first two days of infection are not due to cell death in the monolayers.

3. Effects of IL-1 β , IFN- γ and TNF- α on non-infected SV-HCEC monolayers

SV-HCEC monolayers were seeded in ECIS wells until confluence was reached (see above). The monolayers were then treated with 0.1, 1, 10, 100 and 1,000 ng/ml of TNF- α , IFN- γ , and IL-1 β . The three cytokines were used singly and in combinations. Evaluation of permeability of rickettsiae-infected monolayers in the presence of cytokines was done by infecting cell monolayers with 10 MOI and the addition of the above mentioned cytokines.

Increases in permeability in non-infected SV-HCEC monolayers were dose-dependent when TNF- α was added to the supernatants at concentrations ranging from 0.1 ng/ml to 1,000 ng/ml (Figure 5). At 0.1 ng/ml a mild increase in permeability (5%) was observed during the first 12 hours of infection, after which the monolayer progressively recovered its baseline resistance at 30 hours. At concentrations of 1 ng/ml of TNF- α , a steady increase in permeability was observed which peaked at 50 hours (25% increase) and remained stable thereafter. A minor recovery (less than 5%) was observed towards the end of the experiment (72 hours). At concentrations of 10 ng/ml, a steady increase in permeability was also observed although more pronounced than the increase observed at 1 ng/ml. At 8 hours, an increase of 18% was present which reached 40% by the end of the experiment at 72 hours. At concentrations of 100 and 1000 ng/ml, a very marked increase in permeability was observed at 8 hours

reaching 30 and 45%, respectively. After 8 hours, a steady increase occurred reaching 60 and 70% respectively at 72 hours.

The effects of IL-1 β on SV-HCEC monolayers were also dose-dependent, although the effects on the monolayers were less pronounced when compared to TNF- α (Figure 6). Likewise, the differences in permeability amongst all concentrations were less “linear” when compared to TNF- α . At all concentrations of IL-1 β , a rapid decline was observed at 10 hours, which ranged from 20-30%. A steady recovery of the monolayer’s resistance was observed at doses of 0.1 ng/ml, reaching control values at 72 hours. However, monolayers infected with higher doses showed a steady increase in permeability which reached values between 30-50% at concentrations of 1 ng/ml to 1,000 ng/ml, respectively.

When IFN- γ was added to SV-HCEC monolayers, minor increases in resistance (decreased permeability) were observed and ranged from 2-10% throughout the experiment (Figure 7). No dose dependent effect was observed in any of the monolayers.

4. Effects of TNF- α and IL-1 β on rickettsia-infected SV-HCEC monolayers

Monolayers infected with *R. rickettsii* and treated with TNF- α showed a further increase in permeability when compared to non-treated Rickettsia-infected monolayers and monolayers treated with TNF- α alone in the absence of infection (Figure 8). The increase in permeability was dose-dependent at all time points.

Increases in permeability were almost identical for monolayers treated with 0.1 and 1 ng/ml. In non-infected monolayers, 0.1 ng/ml of TNF- α induced a 10-12% increase in permeability at 6 hours which eventually disappeared at 30-32 hours post-treatment.

However, the permeability in infected monolayers treated with 0.1 ng/ml of TNF- α was increased by 12% at 6 hours (as opposed to 4% in infected and non-treated monolayers). At 12 and 24 hours the differences between treated and non-treated infected monolayers were 12 and 8%, respectively. By 48 hours post-treatment, no differences were noted between TNF- α treated and non-treated monolayers. At TNF- α concentrations of 10 and 100 ng/ml, the effects were more pronounced than at lower concentrations in infected and non-infected monolayers.

Monolayers infected with *R. rickettsii* and treated with IL-1 β showed similar results to the ones obtained with TNF- α (Figure 9).

Experiments performed with Luminex technology have also revealed high concentrations of IL-6 and MCP-1 in *R. rickettsii*-infected monolayers in addition to the three cytokines that we have studied in previous years. Such increase was observed as early as 24 hours post-infection. Because of the effect on permeability that we demonstrated last year caused by supernatants obtained from rickettsia-infected monolayers, we decided to use monoclonal antibodies against IL-6 and MCP-1 in order to assess their effect on endothelial permeability. However, no differences were observed between monolayers treated with supernatants with or without monoclonal antibodies against IL-6 and MCP-1, suggesting that other soluble factor might be involved in increasing endothelial permeability.

5. Immunofluorescence of endothelial monolayers: SV-HCEC were seeded on collagen-coated coverglass and cultured as described above. Following infection with rickettsiae the monolayers were washed and fixed in 4% formaldehyde. After fixation the cells were permeabilized in 0.2% Triton-X 100, blocked with normal goat

serum, and incubated in primary antibody at 4°C overnight. The primary antibodies used were mouse anti-p120 (Zymed), rabbit anti- β -catenin and mouse anti-VE-cadherin (Santa Cruz Biotech). Following a wash step in PBS the cells were incubated in goat-anti-rabbit Alexa-Fluor 488 or goat-anti-mouse Alexa Fluor 594 (Molecular Probes). Finally the cells were counterstained with DAPI and mounted in VECTASHIELD HardSet (Vector Labs). Images were acquired on a Olympus FV-1000 confocal microscope with the 60x objective (N.A. 1.42). Immunofluorescent staining of *R. rickettsii*-infected SV-HCECs has demonstrated loss of p120 staining at intracellular borders within 24 hours of infection and an accumulation of the protein in granular formations within the cytoplasm (Figure 10). Experiments are currently underway to verify these findings. Interestingly we have seen no considerable change in β -catenin staining in response to infection, and this cell line does not appear to express the important adherens junction protein VE-cadherin.

Work on primary mouse brain endothelial cells has demonstrated strong staining for occludin, ZO-1 and VE-cadherin, further confirming the requirement for using primary cells as an *in vitro* model of the blood-brain barrier. Initial experiments with *R. rickettsii*-infected MBECs has demonstrated a frayed appearance of occludin staining after 24 hours of rickettsial infection that correlates with a decrease in electrical resistance across the endothelial monolayer (Figure 11).

6. Effect of exogenous nitric oxide on endothelial permeability

HMEC-1 cultured on 8W10E gold-coated electrodes that were infected with *R. conorii* at an MOI of 10 demonstrated a level of integrity consistent with controls for the first 3 days of infection. After 72 hours we saw a statistically significant drop in

resistance as compared to uninfected controls. Conversely and perhaps most surprisingly, when the endothelial cells were infected with *R. conorii* and also treated with the nitric oxide-donor DETA NONOate at 100uM we saw no statistically significant drop in electrical resistance over the course of the 5 day experiment as compared to uninfected controls. Future experiments are needed to determine if this trend would continue past 5 days. Finally, administration of DETA NONOate at 500uM demonstrated a profound effect on the integrity of the endothelial monolayer. We saw a significant increase in resistance over the first 24 hours of infection and stimulation followed by a slow and steady decline in endothelial integrity as determined by a drop in resistance (Figure 12). Taken together these findings support the hypothesis that nitric oxide can affect the integrity of rickettsiae-infected endothelial monolayers. Furthermore, the use of the peroxynitrite scavenger FetMPyP appeared to partially stabilize the endothelial monolayers to maintain their resistance when compared to monolayers treated only with DETA NONOate (Figure 12).

7. Effect of exogenous nitric oxide on rickettsial gene copy number

HMEC-1 were cultured in 24 well plates and DNA was extracted at 24, 48 and 72 hours of infection and/or stimulation with DETA NONOate. Rickettsial gene copy numbers were normalized to human *gapdh* and copy numbers of rickettsiae between samples were compared using the $\Delta\Delta$ method. We saw no significant change in the numbers of rickettsiae between cells stimulated with and without DETA NONOate for the first 48 hours. However after 72 hours we saw nearly 23 times as many rickettsiae in those cells not stimulated with nitric oxide as compared to those treated at either 100 or 500uM (Table 1). Intriguingly we saw no significant difference

between the ability of either dose of DETA NONOate to control rickettsiae as compared to the other. To confirm that we have introduced NO into the system we tested the cell culture supernatants using the Greiss reaction. We were able to detect high levels of nitrite, a by-product of NO metabolism, at 24 hours after addition of DETA NONOate. The levels maintained themselves through at least the next day of the experiment.

The mechanisms leading to increased microvascular permeability during rickettsial infection have not been extensively studied. There is considerable evidence supporting the role of reactive oxygen species (ROS) as a mechanism of cell damage in response to rickettsial infection. Work by our lab suggests that the presence of ROS may have little effect on rickettsiae-induced changes in tight junction or adherens junction assembly. Instead, the accumulation of ROS may eventually lead to cell death resulting in changes in permeability at late stages of infection. To more accurately reflect the changes that take place in an *in vivo* environment we have decided to pursue a mechanism which has been shown to be an important anti-rickettsial response, and that supports the idea that increased microvascular permeability induced by rickettsiae is in part due to an immunopathological mechanism. Nitric oxide is a well known regulator of vascular tone, vascular remodeling and interendothelial junction assembly. Previous work has also shown that both mouse and human endothelial cells are able to mount an effective anti-rickettsial response when stimulated by certain pro-inflammatory cytokines. The question now lies in how does this response occur in an animal and what effect will that have on the function of the microvasculature.

By introducing NO into this system of rickettsiae-infected microvascular endothelial cells we have been able to expand on the role of NO itself as not only an anti-bacterial effector molecule, but also as a potent regulator of vascular permeability. These studies have revealed that NO is very effective at limiting the number of intracellular rickettsiae. The mechanism of this inhibition however is not well understood at this time. Likewise we have shown that certain lower levels of NO-donors can effectively extend the life-span of endothelial monolayers and has relatively little effect on the barrier function of the monolayer. Conversely higher levels of the NO-donor produce dramatic changes in the integrity of the monolayer by first stabilizing the monolayer and then causing a slow and steady loss of barrier function. Without knowing the degree of NO release in infected endothelial cells *in vivo* at this time we are unable to make conclusions about the definite role of NO as a mechanism leading to increased microvascular permeability. The data presented do at least acknowledge the possibility that this can be the case. However, it also supports the notion that NO may purely be a defense mechanism and that there are other stimuli responsible for the loss of barrier properties. As stated previously a true NO response by way of the iNOS enzyme is dependent on cytokine stimulation of the endothelium. It is likely that cytokine stimulation of the endothelium would cause changes in vascular permeability independent of NO production and would present a challenge in interpreting results in a system this complex. We expect future experiments using primary mouse brain endothelial cells to better describe the true importance of NO as not only an anti-rickettsial defense mechanism but also as a

contributor to disease pathogenesis. In particular, we are interested in the effects of NO on the tight junctions that are distributed ubiquitously throughout the brain. Additionally we have seen an abrogation of rickettsia-induced endothelial permeability in the presence of the broad-spectrum NOS inhibitor L-NAME. We are currently pursuing eNOS as a potential mediator of rickettsia-induced hyperpermeability. In addition we have noticed vast differences in the response of endothelial cells to *R. rickettsii* versus *R. conorii*. Specifically we see a delayed loss of endothelial integrity during *R. conorii* infection when compared to *R. rickettsii*. Additionally we have seen a small increase in supernatant nitrite levels during *R. rickettsii* infection that is not present during *R. conorii* infection. Whether this small increase in nitric oxide production is of any significance with respect to microvascular permeability is a point of current investigation.

8. Calcium signaling

We have established a protocol to transfect SV-HCEC with the calmodulin activation indicator Yc2.1. Lipofectamine with Plus Reagent has proven to be a suitable tool for the transfection of this cell line. Initial experiments have demonstrated strong expression of the vector within 48-72 hours, however the transfection efficiency appears to be relatively low in this cell line (<50%, Figure 13). Preliminary data obtained by infecting Yc2.1-transfected SV-HCEC with *R. rickettsii* has shown no considerable change in the ratio between CFP and YFP emission intensities within the initial hours of infection.

Similarly, SV-HCEC loaded with 2uM of fluo-3 and fura-red dyes have shown no considerable change intracellular calcium levels in response to rickettsial infection

(Figure 14). We are currently optimizing the confocal microscope settings to most efficiently image and detect the fluorescent signals while minimizing the level of photobleaching over the course of several hours of imaging.

III. KEY RESEARCH ACOMPLISHMENTS

Successful differentiation of a murine monocytic cell line (M1) to macrophage lineage followed by successful transfection with adaptein-containing retroviruses.

Successful use of primary brain microvascular endothelial cells of murine origin in permeability experiments using ECIS.

Expansion of cytokine studies on endothelial monolayers infected with *R. rickettsii*.

Expansion of studies related to the role of nitric oxide and peroxynitrite in modulation of endothelial barrier functions.

Demonstration of the role of p120 in adherens junctions changes in SV-HCEC monolayers.

Demonstration of the role of occluding in tight junction changes in primary murine brain endothelial cells.

IV. REPORTABLE OUTCOMES

Publications

1. Woods ME, Koo P, Wen G, **Olano JP**. Nitric oxide (NO) as a mediator of increased microvascular permeability during rickettsial infections. Annals of the New York Academy of Sciences. 2005;1063:239-245.
2. **Olano JP**. Rickettsial infections. Annals of the New York Academy of Sciences. 2005;1063:187-196.

Abstracts

1. **Olano JP**. Permeability studies on *Rickettsia rickettsii*-infected human cerebral microvascular endothelial cells. Department of Defense Military Health Research Forum. Puerto Rico. 2006.

2. Woods ME, **Olano JP**. Peroxynitrite-mediated damage during rickettsial infection of human microvascular endothelial cells. American Society of Investigative Pathology. San Francisco. 2006.
3. Koo P, Woods ME, **Olano JP**. *In vitro* studies of microvascular permeability during *Rickettsia rickettsii* infections. International Conference on Rickettsiae and Rickettsial Diseases. Abstract Book. 2005:O23
4. **Olano JP**. Rickettsial infections. International Conference on Rickettsiae and Rickettsial Diseases. Abstract Book. 2005:L3
5. Woods ME, Koo P, Wen G, **Olano JP**. Nitric oxide (NO) as a mediator of increased microvascular permeability during rickettsial infections. International Conference on Rickettsiae and Rickettsial Diseases. Abstract Book.2005:O59.

V. CONCLUSIONS

Several goals were accomplished during the fourth year of this project. A no-cost extension was granted for one year in order to finalize experiments primarily related to the first specific aim of the project and final experiments related to calcium signaling in specific aim 3.

The role of rickettsial organisms and cytokines in endothelial permeability is now well defined. Both IL-1 β and TNF- α have a profound effect in endothelial permeability which is synergistic with the presence of very low numbers of rickettsia in the cytoplasmic compartment. IFN- γ seems to have a protective effect regarding increased permeability across the endothelial monolayers. The role of nitric oxide in rickettsial infections is both beneficial and detrimental. On the one hand, low concentrations of nitric oxide seems to control the proliferation of intracellular rickettsiae and no effects on endothelial permeability are observed. However, at high concentrations, rickettsial numbers are controlled intracellularly, but dramatic effects are observed in permeability of endothelial monolayers. The use of peroxynitrite

scavengers stabilizes the resistance across cell monolayers, implying that both ROS and NO interact inside the cell to form peroxynitrite which in turn induces permeability changes.

Both p120 in adherens junctions and occluding in tight junctions seem to play a role in permeability changes observed in rickettsia-infected monolayers. Studies are currently underway to better characterized these changes at the biochemical level.

VI. SCIENTIFIC PERSONNEL

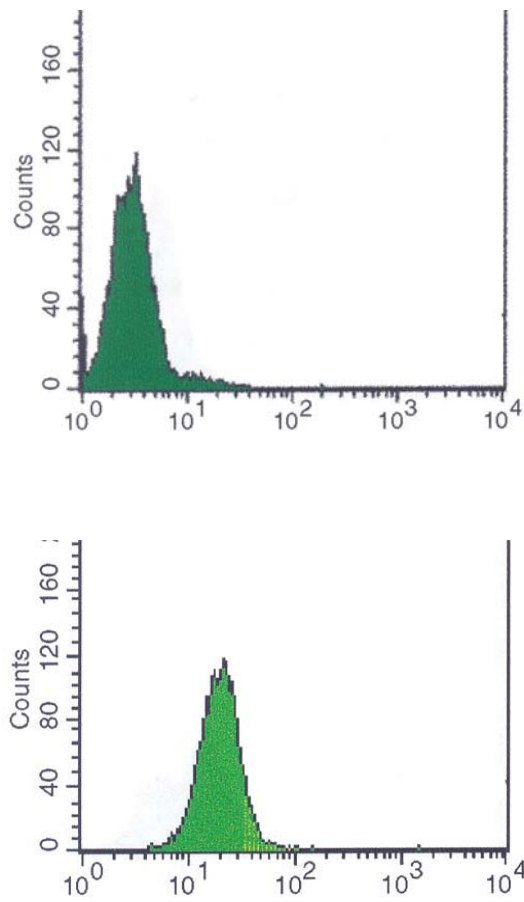
1. David H. Walker, M.D. Principal Investigator (10% effort)
2. Juan P. Olano, M.D. Co-Principal Investigator (25% effort)
3. Michael Woods, B.Sc. Graduate student (100% effort).
4. Gary Wen, M.Sc. (50% effort)
5. Leoncio Vergara, M.D. (10% effort, as of June 2004).

VII. REFERENCES

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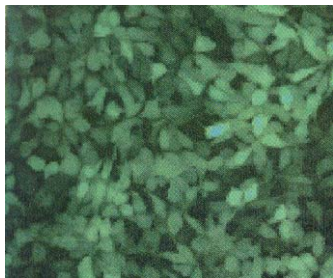
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Figure 1



Top: M1 cells. CD11b expression is negative. Bottom: M1 cells treated with 10^{-5} M of dexamethasone for 5 days. CD11B expression is strong and uniform.

Figure 2



EGFP expression of differentiated M1 cells after infection with pantropic retroviruses

Figure 3

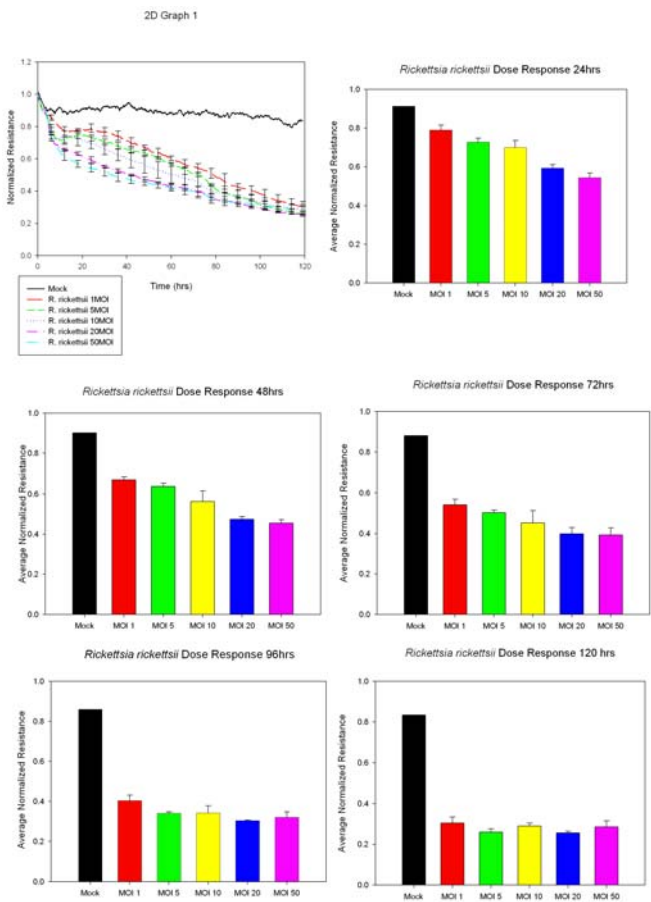
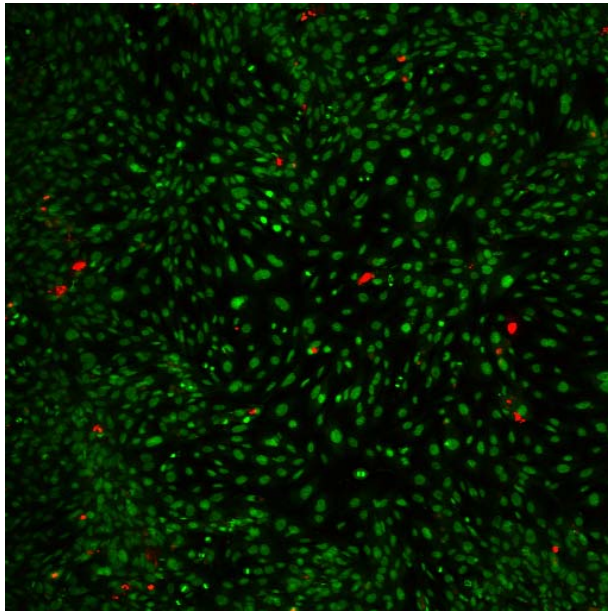
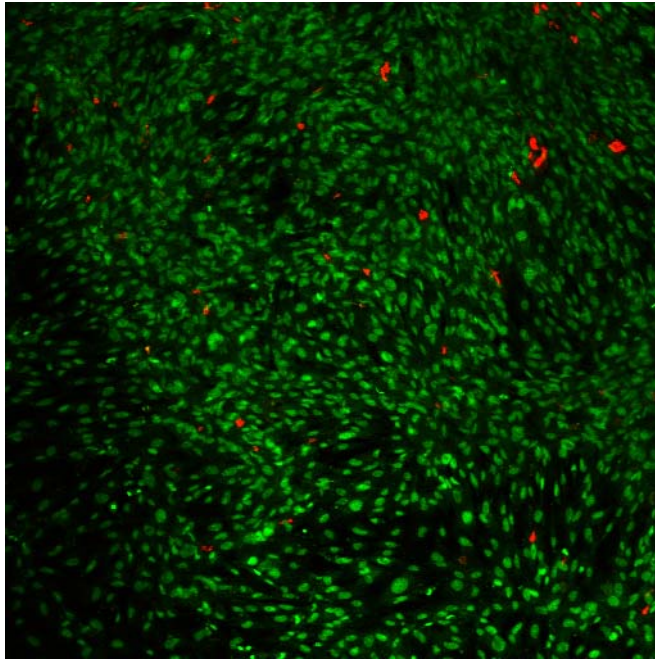


Figure 4



Top: Mock infected SV-HCEC monolayers with scattered PI-positive cells at 48 hours post-infection. Bottom: SV-HCEC cells infected with 15 MOI of *R. rickettsii* for 48 hours. Scattered PI-positive cells in the field.

Figure 5

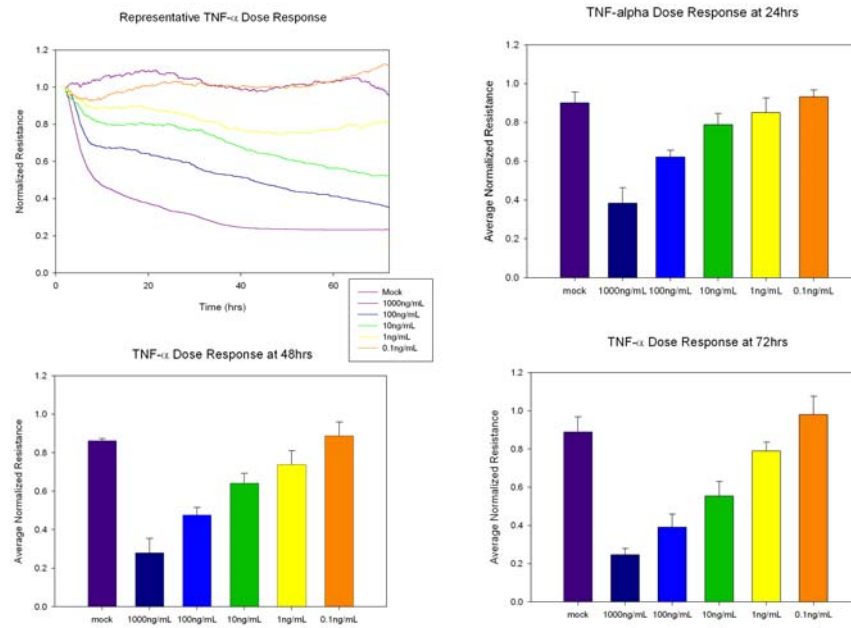


Figure 6

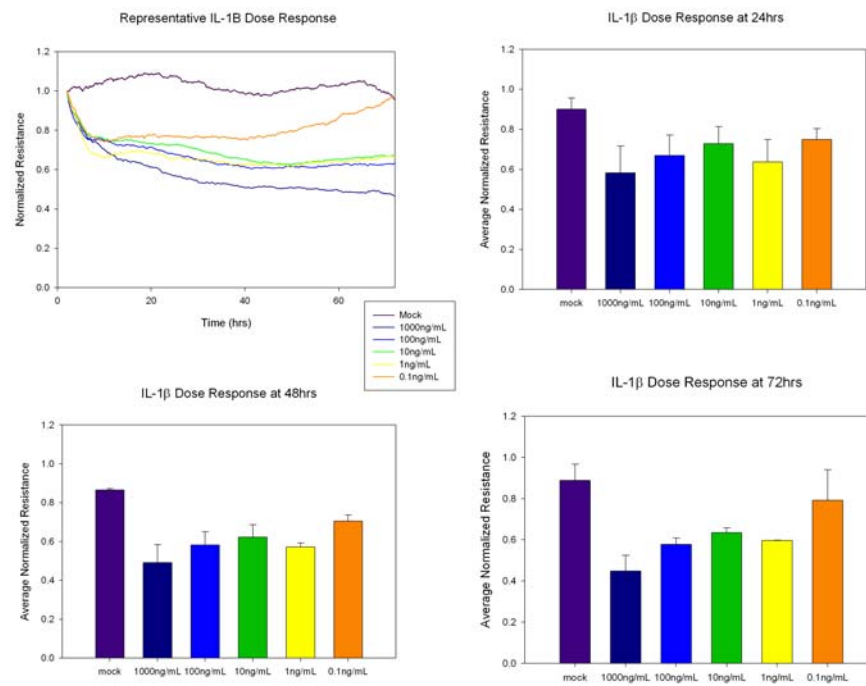


Figure 7

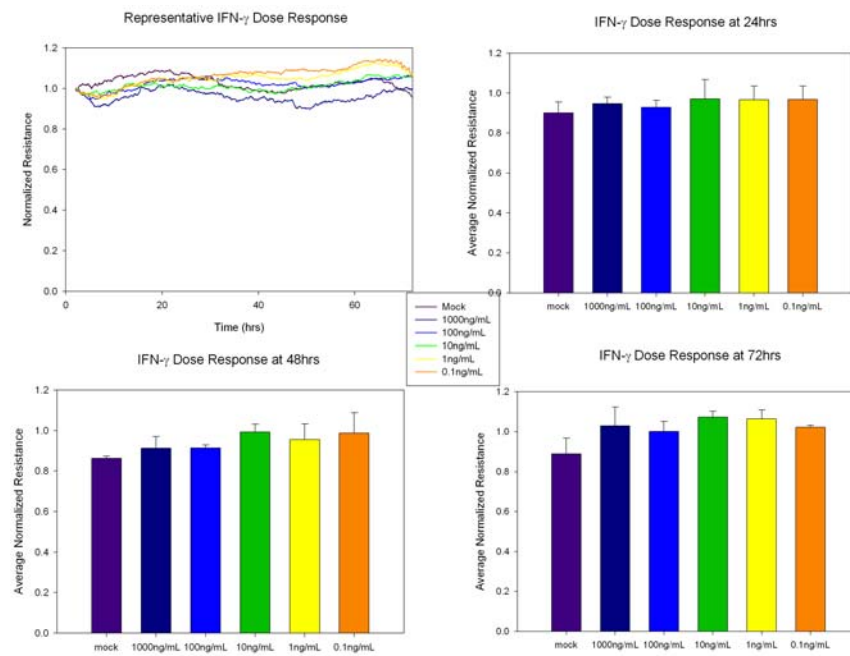


Figure 8

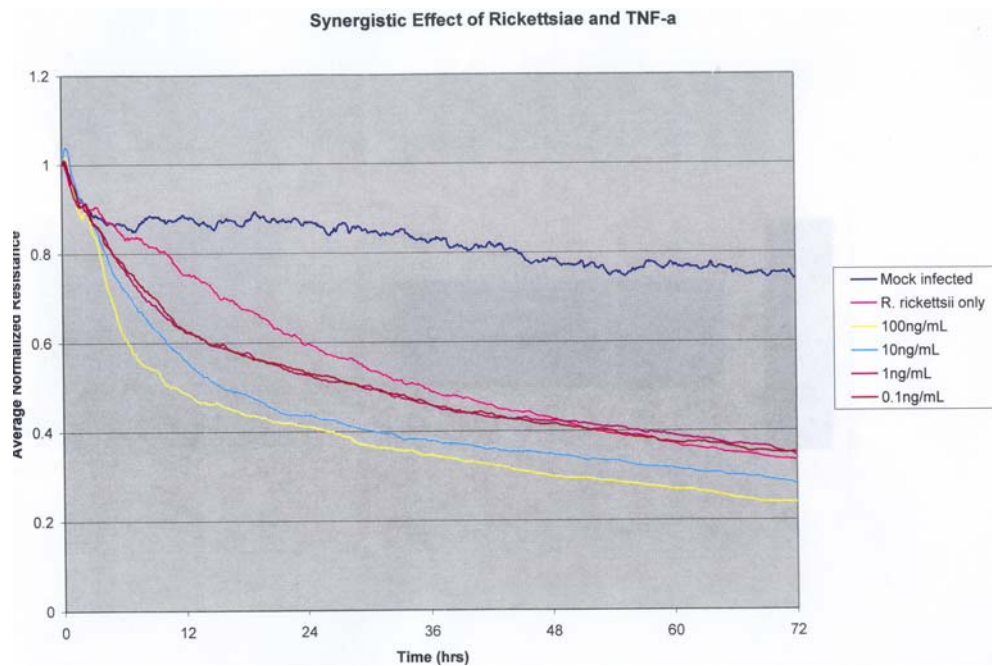


Figure 9

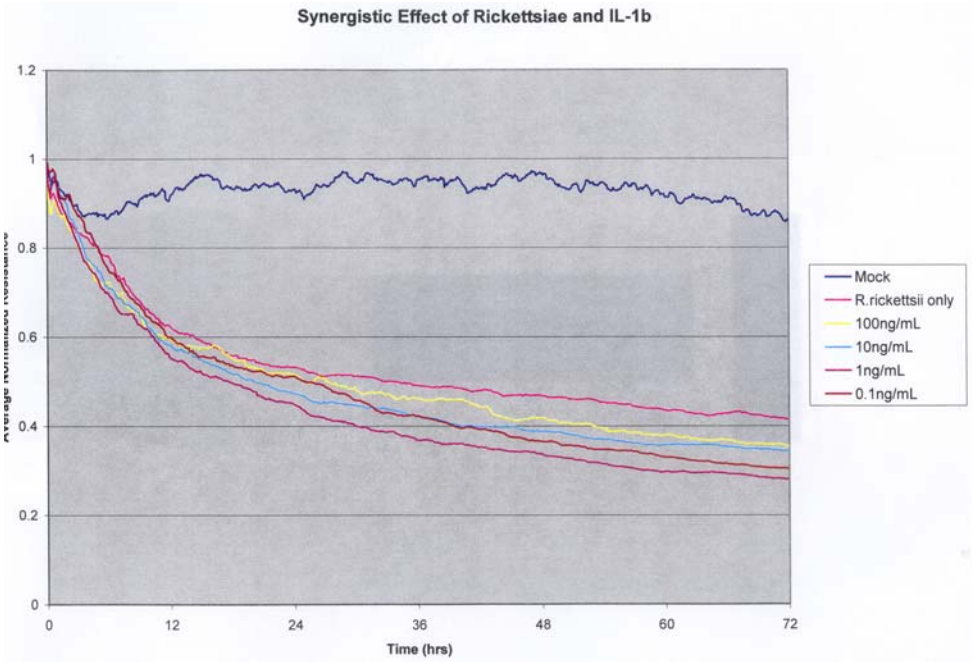


Figure 10

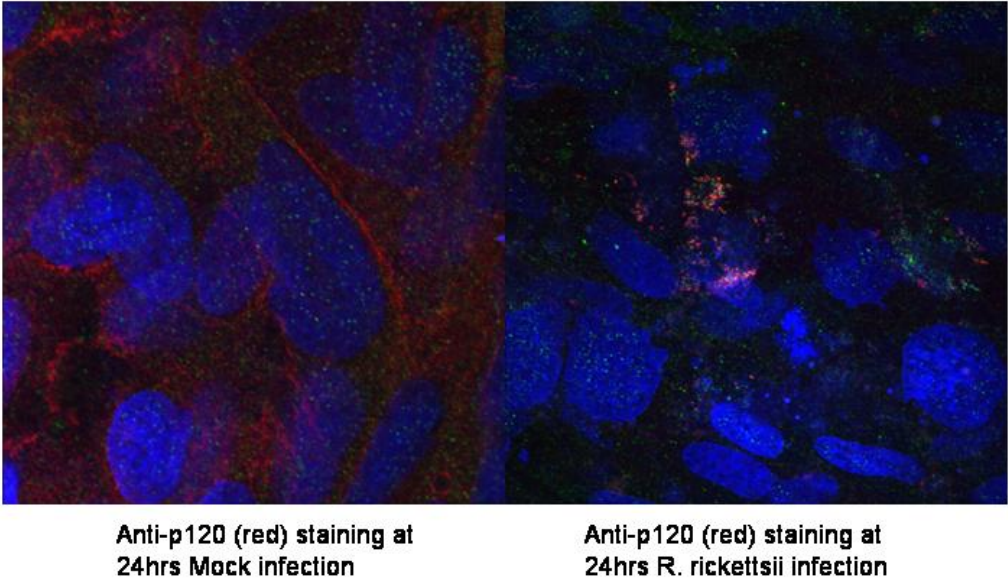
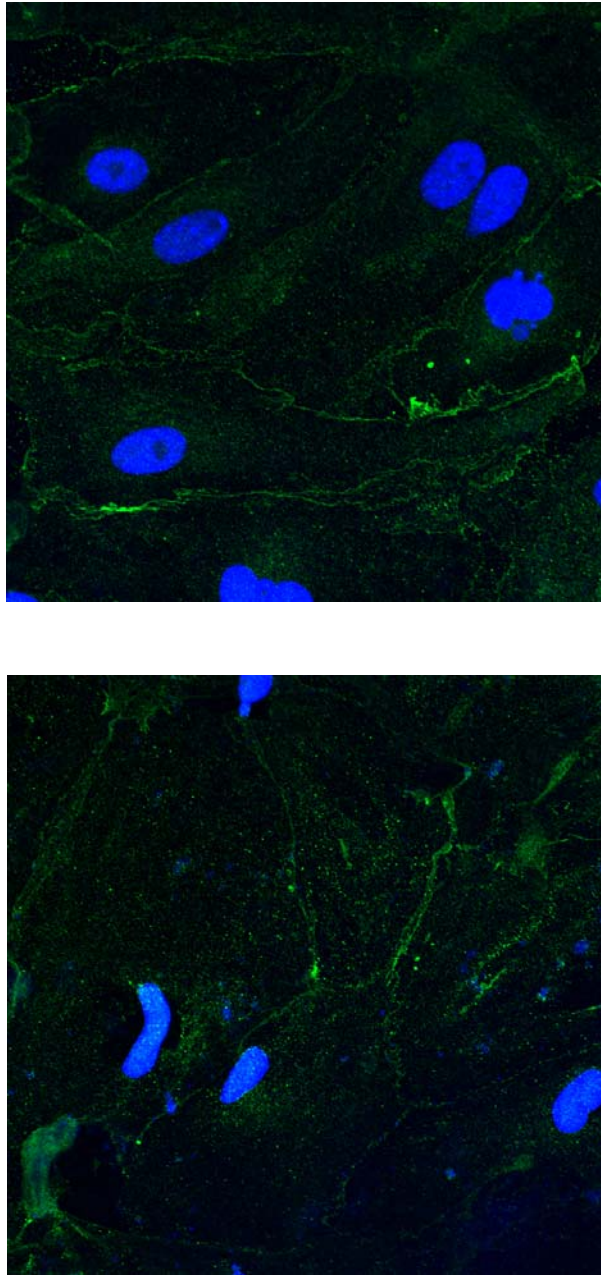


Figure 11



Primary murine brain microvascular endothelial cells stain with anti-occludin antibodies. Top: Mock infected at 24 hours. Bottom: Infected with *R. rickettsii* for 24 hours. Note “frayed” appearance of occluding membrane staining in infected cells. DAPI was used as nuclear counterstain.

Figure 12

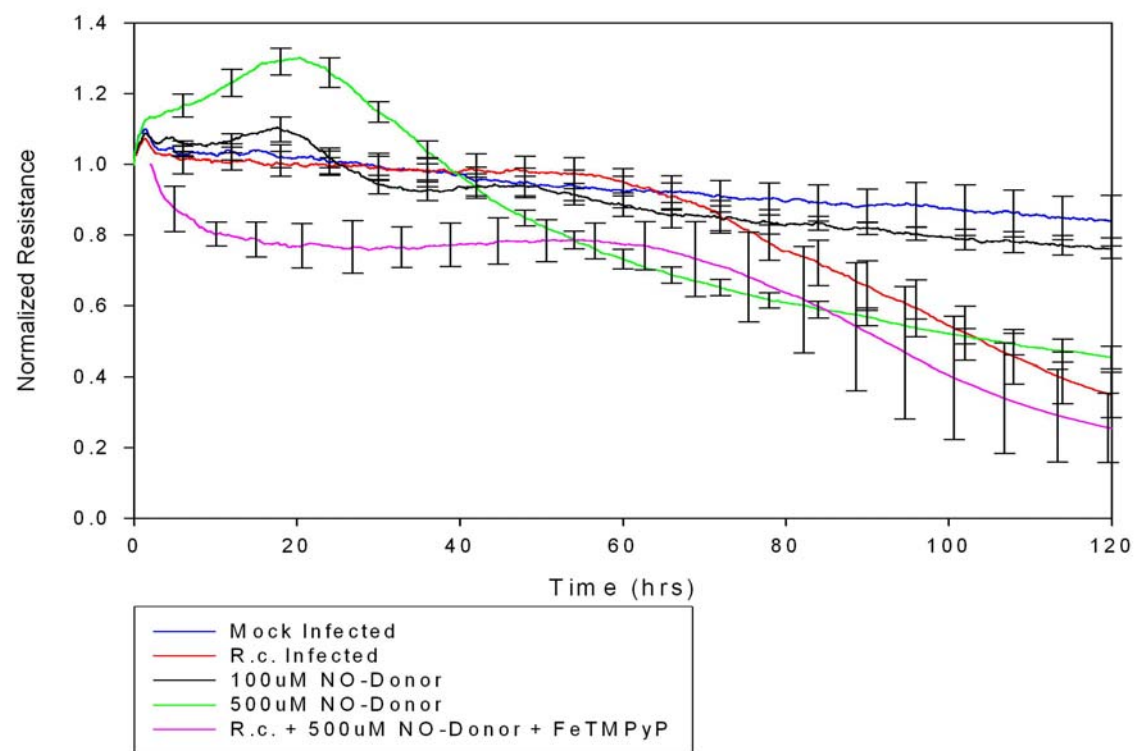
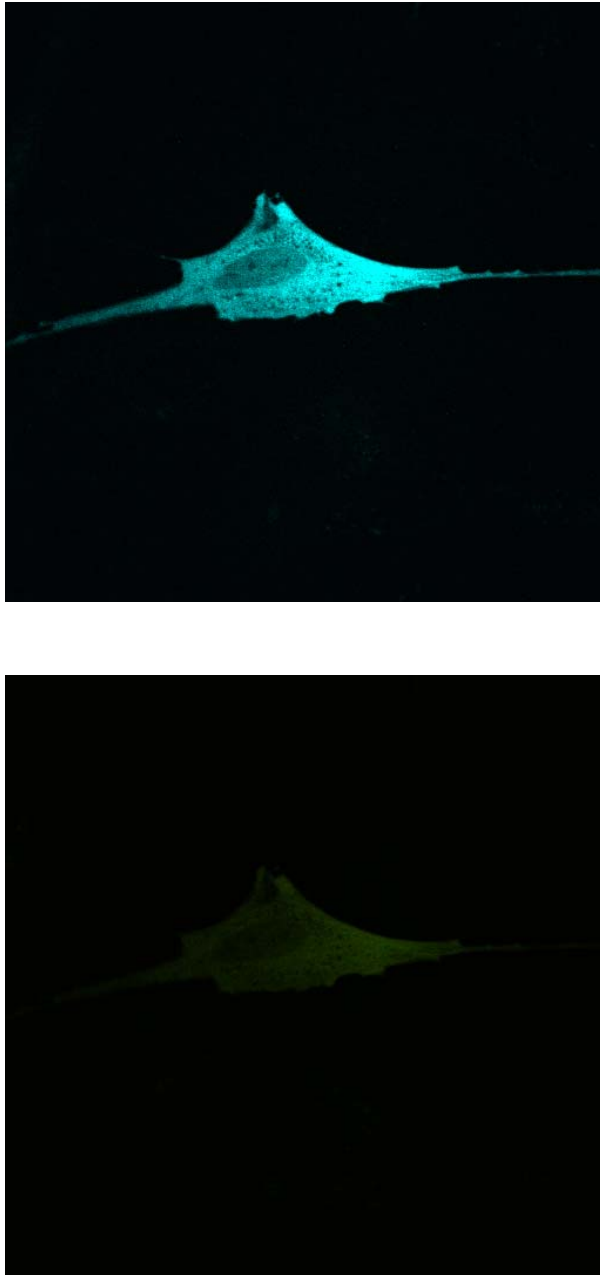
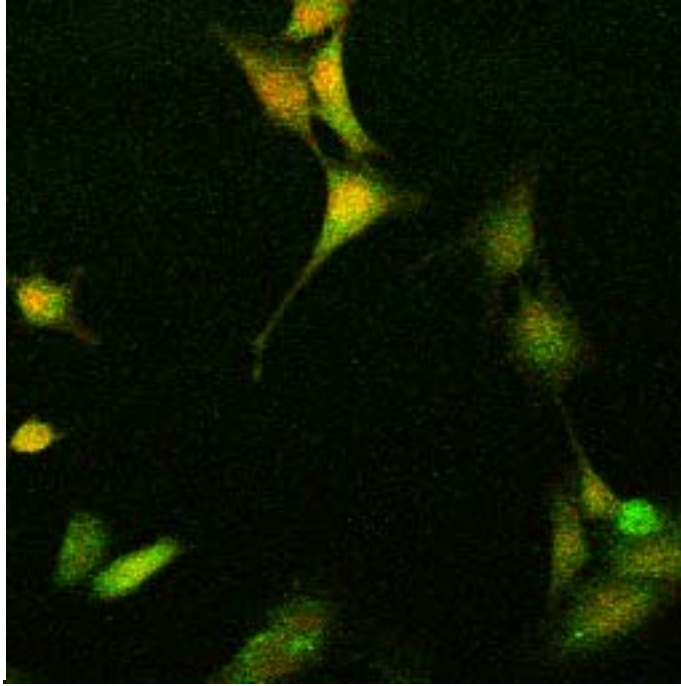


Figure 13



Top: SV-HCEC transfected with plasmid Yc2.1 for detection of calmodulin activation. CFP emission. Bottom: YFP emission. Ratiometric measurements have shown minor changes after infection.

Figure 14



Fluo 3 emission is green mixed with Fura Red emission in SV-HCEC cells.

Table 1

	<i>R. conorii</i> / <i>R. conorii</i> + DETA NONOate (100uM)	<i>R. conorii</i> / <i>R. conorii</i> + DETA NONOate (500uM)
24 hrs	1.76±0.98 X	1.15±0.17 X
48 hrs	3.34±1.99 X	2.00±0.90 X
72 hrs	23.5±8.99 X	23.45±10.54 X

Table 1. Exogenous nitric oxide limits the proliferation of intracellular *R. conorii*. The effect is not noticed until 3 days after infection when there were significantly fewer rickettsiae as measured by real-time PCR for the rickettsial citrate synthase gene, *gltA*. Relative gene copy number was normalized to *hgapdh* and compared using the $\Delta\Delta$ method. Data indicates fold change in relative copy numbers compared to untreated, infected cells.